Insulin and insulin-like growth factor I (IGF-I) stimulate GLUT4 glucose transporter translocation in *Xenopus* oocytes

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1. The heterologous expression of glucose transporters GLUT4 and GLUT1 in *Xenopus* oocytes has been shown to cause a differential targeting of these glucose-carrier isoforms to cellular membranes and a distinct induction of glucose transport activity. In this study we have evaluated the effect of insulin and insulin-like growth factor I (IGF-I) on glucose uptake and glucose transporter distribution in *Xenopus* oocytes expressing mammalian GLUT4 and GLUT1 glucose carriers. 2. Insulin and IGF-I stimulated 2-deoxyglucose uptake in GLUT4-expressing oocytes, but not in GLUT1-expressing oocytes or in water-injected oocytes. The stimulatory effect of insulin and IGF-I on 2-deoxyglucose uptake in GLUT4-expressing oocytes occurred via activation of the IGF-I receptor. 3. Subcellular-fractionation studies indicated that insulin and IGF-I stimulated translocation of GLUT4 to the cell surface of the oocyte. 4. Incubation of intact oocytes with insulin stimulated phosphatidylinositol 3-kinase activity, an effect that was blocked by the additional presence of wortmannin. Furthermore, wortmannin totally abolished the insulin-induced stimulation of 2-deoxyglucose uptake in GLUT4-expressing oocytes. 5. In this study, both the insulin-induced GLUT4 carrier translocation and GLUT4-dependent insulin-stimulated glucose transport have been reconstituted in the *Xenopus* oocyte. These observations, together with the fact that wortmannin, as found in adipocytes, inhibits insulin-stimulated glucose transport in oocytes, suggest that the heterologous expression of GLUT4 in oocytes is a useful experimental model by which to study the cell biology of insulin-induced GLUT4 translocation.

INTRODUCTION

Muscle and adipose cells respond acutely to insulin by stimulation of glucose uptake. This process is mediated by a rapid increase in cellular glucose transport resulting from the redistribution of glucose carriers from an intracellular compartment to the cell surface [1,2]. Glucose transport in muscle and adipose cells is performed by GLUT1 and GLUT4 carriers, the latter accounting for over 95% of cellular glucose carriers [3,4].

GLUT4 carriers under basal conditions are mainly intracellular, and insulin causes the translocation of GLUT4 from an intracellular site to the cell surface in adipocytes, cardiomyocytes and muscle fibres [3,5–9]. Since they are more abundant, GLUT4 transporters are responsible for most of the stimulation of glucose transport observed in response to insulin. In contrast, GLUT1 carriers are present in higher proportion in the plasma membrane of basal cells [10]. However, some GLUT1 is intracellular, and it also undergoes insulin-stimulated movement to the cell surface in adipocytes and cardiomyocytes [3,11,12].

The expression of GLUT1 and GLUT4 in various non-insulin-sensitive mammalian cell types leads to a differential induction of glucose transport and to a specific subcellular distribution of carriers. Thus, transfection of cells with GLUT1 causes a marked increase in basal glucose uptake, and a substantial amount of GLUT1 is found in the plasma membrane [13–16]. In contrast, GLUT4 transfection in an insulin-insensitive cell type does not increase basal glucose transport, and GLUT4 is only found in an intracellular compartment [13–16]. Furthermore, the presence of GLUT4 alone does not allow the cell to respond to insulin by recruiting GLUT4 to the cell surface [13,14,16,17].

The microinjection of GLUT1 and GLUT4 in *Xenopus* oocytes leads to a similar pattern to that obtained after transfection of glucose carriers in mammalian cells. Thus, injection of GLUT1 cRNA induces greater glucose transport activity in oocytes than the injection of GLUT4 cRNA [18,19], and GLUT1 is more efficiently targeted to the plasma membrane of the oocyte than GLUT4 [20]. Based on the observation that, under some conditions, oocytes respond to insulin and insulin-like growth factor I (IGF-I) by activating glucose uptake [21–23], we have explored whether these hormones stimulate GLUT4 and/or GLUT1 activity when expressed in *Xenopus* oocytes.

METHODS

Materials

^{125}I-Protein A, [γ-^{32}P]ATP and ^{125}I-labelled sheep anti-mouse antibody were purchased from ICN. 2-Deoxy[^{3}H]glucose was obtained from New England Nuclear. Immobilon was obtained from Millipore. All electrophoresis reagents and molecular-mass markers were obtained from Bio-Rad. Wortmannin was kindly given by Dr. Trevor Payne (Sandoz, Basel). Human recombinant IGF-I was from Ciba–Geigy, and bovine recombinant insulin was from Lilly Research Laboratories. Human GLUT1 and rat GLUT4 cDNAs were kindly given by Dr. Graeme I. Bell (University of Chicago) and Dr. Morris Birnbaum (Harvard University). γ-Globulin, phosphatidylinositol (PI), its 3- and 4-phosphate (PI-3-P, PI-4P) and most commonly used chemicals were from Sigma.

Antibodies

Both monoclonal (1F8) and polyclonal (OSCRX) antibodies specific for GLUT4 were used in these studies. Monoclonal antibody 1F8 [5] was kindly given by Dr. Paul F. Pilch (Boston University). Anti-GLUT4 antibody (OSCRX) from rabbit was

Abbreviations used: IGF-I, insulin-like growth factor I; PI, phosphatidylinositol; PI-3-P, PI 3-phosphate; PI-4-P, PI 4-phosphate.

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produced after immunization with a peptide corresponding to the final 15 amino acids of the C-terminus [24]. A polyclonal antibody generated against the C-terminus of GLUT1 obtained from East Acres was used. Monoclonal antibody 8C8 against *Xenopus* β_1-integrin [25] was kindly given by Dr. Peter Hausen (Max-Planck Institüt für Entwicklungsbiologie, Tübingen). Monoclonal antibody Cl 10.1 against synaptobrevin (VAMP-1) [26] was kindly provided by Dr. Reinhard Jahn (Howard Hughes Medical Institute, Boyer Center for Molecular Medicine, New Haven). The monoclonal antibody against phosphotyrosine was obtained from UBI (New York).

Oocytes, injections and glucose-uptake measurement

Xenopus laevis females were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, Germany). Small clumps of oocytes were treated with collagenase D (Boehringer Mannheim) at 2 mg/ml in a Ca^{2+}-free solution (ORII: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes/Tris, pH 7.5) for 2 × 90 min to remove the follicular layer as previously reported [27]. After thorough washing in ORII solution and modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes/Tris, pH 7.5], the oocytes were kept in modified Barth's solution overnight at 18 °C. After this incubation period, healthy-looking Stage VI oocytes were injected with different amounts of cRNA (0–25 ng of RNA) corresponding to human GLUT1 or rat GLUT4 (i.e. cDNA transcribed *in vitro*, as described below) or diethyl pyrocarbonate-treated water. RNA samples were dissolved in diethyl pyrocarbonate-treated water at 0.2–0.3 μg/μl. RNA samples or water were injected into oocytes by a semi-automatic injector (Inject + Matic-system, J. A. Gabay, Geneva). The volume injected was 50 nl. Oocytes were then incubated at 18 °C for various times (3–6 days) in modified Barth's solution containing gentamycin sulphate (20 mg/l).

On the day of the experiment, groups of 7–10 oocytes were incubated for 60 min with or without different concentrations of insulin (0–1 μM) or IGF-I (0–100 nM) in 90 μl of Barth's solution. After this time, and to determine uptake, 90 μl of Barth's solution containing 2-deoxy[^{3}H]glucose was added to the incubation medium to a final concentration of 2-deoxyglucose of 50 μM at 2 μCi/ml. Uptakes were performed at 25 °C for 45 min. Preliminary time-course experiments showed linearity up to 90 min in oocytes expressing GLUT4 or GLUT1, and in uninjected oocytes (results not shown). After incubation, the uptake solution was removed and the oocytes were washed in 3 × 4 ml of ice-cold PBS containing 100 mM D-glucose and 0.1 mM phloretin. In some experiments, the relative proportion of intracellular 2-deoxyglucose in free and phosphorylated forms was assessed by ion-exchange chromatography. For this purpose, lysates were prepared by homogenizing 40 oocytes in 2 ml of 6% $HClO_4$ in a Polytron (30 s, setting 5). The homogenate was centrifuged at 5000 rev./min for 15 min and the supernatant was neutralized in 2 M KOH/0.5 M triethanolamine. A portion of neutralized lysate (1.5 ml) was applied to a 1 ml column of AG 1X (acetate form). 2-Deoxy[^{3}H]glucose that had not reacted was eluted with 3 ml of water. The phosphorylated sugar was subsequently eluted with 3 ml of 6% $HClO_4$. Samples of the eluted solutions were taken for scintillation counting. Unspecific uptake of hexoses was determined by using labelled L-glucose under similar conditions, as explained for 2-deoxyglucose. Immediately, each single oocyte was put into a scintillation vial and dissolved in 200 μl of 10% SDS, and the radioactivity was counted after addition of 3 ml of scintillation fluid.

PI 3-kinase activity

Before PI 3-kinase assay, oocytes were incubated in Barth's solution and treated or not with 1 μM wortmannin for 30 min at 18 °C before adding insulin (or not) to a final concentration of 1 μM during 30 min at 18 °C. Oocytes were then solubilized for 30 min at 4 °C in a buffer containing 50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM vanadate, 0.5 mM PMSF, 2 μM leupeptin and 2 μM pepstatin, supplemented with 1% Nonidet P40. The solubilized preparations were centrifuged at 10000 *g* for 20 min at 4 °C and the supernatants were submitted to immunoprecipitation with the indicated antibodies. Monoclonal anti-phosphotyrosine antibodies (7 μg) or the corresponding non-immune controls were preadsorbed on Protein-G–Sepharose at 4 °C during 1 h and washed twice with 30 mM Hepes/30 mM NaCl/0.1% Triton X-100, pH 7.4, before being incubated with 2.5 mg of the solubilized proteins for 90 min at 4 °C. The immunoprecipitates were washed twice with each of the three following buffers: (a) PBS, pH 7.4, containing 1% Nonidet P40; (b) 100 mM Tris/0.5 M LiCl, pH 7.0; and (c) 10 mM Tris/100 mM NaCl/1 mM EDTA, pH 7.4 [28,29]. The pellets were resuspended in 30 μl of 20 mM Hepes/0.4 mM EDTA/0.4 mM Na_2HPO_4. The substrate (PI) was dried for 10 min, resuspended in 5 mM Hepes at 1 mg/ml and sonicated for 15 min. The kinase reaction was started by addition of PI at a final concentration of 0.2 mg/ml, 10 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). After 15 min the reaction was stopped by addition of 15 μl of 4 M HCl, and the phosphoinositol lipids were extracted with 130 μl of chloroform/methanol (1:1, v/v). The tubes were mixed by vortex-mixing for 30 s and centrifuged for 1 min [28,30]. The organic phase was recovered, dried, and the phospholipids were resuspended in 5 μl of chloroform and separated by TLC on silica-gel 60A in chloroform/methanol/4.3 M ammonia (9:7:2, by vol). The plate was analysed by autoradiography. The utilization of PI-4-P standard confirmed the position in the plate of ^{32}P-labelled PI-3-P (results not shown).

Synthesis of transcripts from cDNA (cRNA)

GLUT1-(pGEM4Z) and GLUT4-(pSM111) containing plasmids were isolated by using a miniprep kit (Promega). The plasmid was linearized by *Bam*HI restriction-endonuclease digestion and transcribed *in vitro*, using T7 and T3 RNA Polymerase (Promega) in the presence of ^{7}mGpppG (NEB), as described elsewhere [31]. Briefly, 1–2 μg of linearized plasmid DNA was incubated in 1 × transcription buffer supplied with the enzyme (Promega), 0.5 mM ATP, CTP, UTP and ^{7}mGpppG, 10 mM dithiothreitol, 0.1 mM GTP, 1 unit/μl of RNA-guard (Pharmacia) and 30 units of T7 or T3 RNA polymerase (Promega) in a final volume of 50 μl. After incubation for 1 h at 37 °C, DNA was digested with 10 units of DNase I (Boehringer Mannheim) and 50 units of RNA-guard. RNA was extracted twice with phenol/chloroform (1:1, v/v) and precipitated with ammonium acetate and ethanol. The cRNA was finally resuspended in 10 μl of diethyl pyrocarbonate-treated water, and a sample was quantified by its A_{260}, and transcript integrity was checked on a 1%-agarose/formaldehyde gel.

Preparation of total membranes from oocytes

Whole membranes were isolated from oocytes as described by García et al. [32]. For this, 20–25 oocytes were lysed in 200 μl of a solution containing 250 mM sucrose, 0.021 mM leupeptin and 1 mM PMSF by using a Gilson P-200 Pipetteman. The triturated

oocytes were further homogenized in a Microfuge tube with a Teflon pestle (20 strokes). Homogenates were centrifuged in a Microfuge at 13000 rev./min for 15 min. The supernatant was again centrifuged at 13000 rev./min for the same time. The supernatants were collected and stored at −20 °C. Proteins were measured by the method of Bradford [33], with γ-globulin as standard.

Subcellular fractionation of oocytes

Membrane fractions enriched in plasma membrane and in intracellular membranes from oocytes were obtained as described previously [34]. Then 25 oocytes were placed in a small Petri dish containing 1 ml of ice-cold homogenization buffer, of composition 10 mM Hepes, 83 mM NaCl, 1 mM $MgCl_2$, 1 μg/ml pepstatin A, 1 μg/ml leupeptin and 100 μM PMSF, pH 7.9. Oocytes were gently squeezed, and oocyte ghosts were separated from the rest of the cellular contents. Oocyte ghosts were placed in 1 ml of ice-cold homogenization buffer supplemented with 0.5 mM NaCl and stirred on ice for 10 min. Ghosts were then centrifuged for 10 min at 1000 ***g***.

The pellet was resuspended in 30 mM Hepes/250 mM sucrose, pH 7.4, and referred to as fraction F1; it contained melanosomes. The supernatant from the centrifugation was centrifuged at 10000 ***g*** for 20 min. The resulting pellet was resuspended in 30 mM Hepes/250 mM sucrose, pH 7.4, and was found to be enriched in plasma-membrane components (fraction F2). The supernatant of the 10000 ***g*** spin was combined with the cellular debris initially obtained, and subjected to repeated spins at 1000 ***g*** for 10 min to pellet the yolk proteins. The supernatant of the centrifugation was centrifuged at 100000 ***g*** for 60 min, and the pellet from this centrifugation contained intracellular membranes (fraction F3). All fractions were resuspended in 30 mM Hepes/250 mM sucrose, pH 7.4, and stored at −20 °C. Proteins were measured by the method of Bradford [33] with γ-globulin as standard.

Electrophoresis and immunoblot analysis

SDS/PAGE was performed on membrane protein by the method of Laemmli [35]. Proteins were transferred to Immobilon as previously reported [36] in buffer consisting of 20% methanol, 200 mM glycine and 25 mM Tris, pH 8.3. After transfer, the filters were blocked with 5% non-fat dry milk/0.02% NaN_3 in PBS for 1 h at 37 °C, and then incubated with antibodies. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Polyclonal antibody OSCRX (raised against the 15C-terminal peptide from GLUT4) was used at 1:400 dilution in 1% non-fat dry milk/0.02% NaN_3 in PBS overnight at room temperature, to immunoblot GLUT4. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished by using ^{125}I-Protein A for 4 h at room temperature or with an enhanced chemiluminescence (ECL) system (Amersham Corp). The autoradiograms were quantified by scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear-response range.

RESULTS AND DISCUSSION

Insulin and IGF-I stimulate glucose transport in GLUT4-expressing oocytes

cRNAs of GLUT1 or GLUT4 were injected into oocytes and the induction of 2-deoxyglucose uptake was evaluated. Time-course experiments indicated that a maximal induction of glucose uptake was obtained 2 days after GLUT1 or GLUT4 cRNA injection (results not shown). Experiments were also performed to evaluate the dependence of induction of 2-deoxyglucose on the amount of cRNA injected. At 5 days after injection of different amounts of cRNA of GLUT1 or GLUT4, ranging from 0 to 25 ng, maximal levels of 2-deoxyglucose uptake were obtained after injection of approx. 6 ng of cRNA for both GLUT1 and GLUT4 (results not shown). Injection of 10 ng of GLUT1 cRNA led to a near 100-fold increase in 2-deoxyglucose uptake over basal activity (Table 1). Injection of GLUT4 cRNA led to a lower induction of 2-deoxyglucose uptake compared with that obtained after GLUT1 cRNA injection (Table 1). These results indicate a differential level of induction of 2-deoxyglucose uptake after injection into oocytes of cRNA for GLUT1 and GLUT4, which is in keeping with previous observations [18,19].

Table 1 Insulin and IGF-1 stimulate glucose transport in GLUT4-expressing oocytes

Oocytes were injected with water or with 10 ng of cRNA for GLUT1 or GLUT4. At 5 days after the injection, oocytes were incubated for 60 min in the absence or presence of 1 μM insulin or 100 nM IGF-I. After this time, 2-deoxyglucose uptake was determined for 45 min in the presence of 50 μM 2-deoxy[^{3}H]glucose at 5 μCi/ml. Results are means ± S.E.M. obtained from seven or ten oocytes per group of a representative experiment from six to ten separate experiments; * indicates a significant difference due to insulin or IGF-1 treatment, at $P < 0.05$. Basal 2-deoxyglucose uptake was 0.79 ± 0.06 pmol/45 min per oocyte ($n = 20$ independent experiments). The expressed 2-deoxyglucose uptake in GLUT4-expressing oocytes (uptake in GLUT4-expressing oocytes minus the basal) was 3.49 ± 0.63 pmol/45 min per oocyte ($n =$ 12 independent experiments), whereas in GLUT1-expressing oocytes it was 74.70 ± 8.27 pmol/45 min per oocyte (n = 8 independent experiments).

	2-Deoxyglucose uptake (pmol/45 min per oocyte)	
	Basal	Insulin
Water-injected	0.36 ± 0.11	0.34 ± 0.09
GLUT4	1.97 ± 0.27	4.61 ± 0.59*
GLUT1	34.5 ± 1.3	36.0 ± 3.1
	Basal	IGF-1
Water-injected	0.59 ± 0.04	0.67 ± 0.04
GLUT4	1.42 ± 0.16	2.58 ± 0.21*
GLUT1	36.0 ± 1.7	42.6 ± 1.9

The effect of insulin and IGF-I on the uptake of 2-deoxyglucose in water-injected, GLUT4-expressing and GLUT1-expressing oocytes was next explored. For this purpose, oocytes were incubated for 1 h in the absence or presence of 1 μM insulin or 100 nM IGF-I, and after this time 2-deoxyglucose uptake was assayed (Table 1). Under these conditions, no effect of these agents was found on glucose uptake in water-injected oocytes (Table 1). Furthermore, no effect of insulin or IGF-I was detected in GLUT1-expressing oocytes (Table 1). However, in GLUT4-expressing oocytes a 2–3-fold increase in 2-deoxyglucose uptake was found (Table 1). The effect of insulin and IGF-I on 2-deoxyglucose uptake was specific, and no effect on L-glucose uptake was detected (results not shown). Furthermore, the effect was a consequence of the glucose transport process, and the intracellular concentrations of free and phosphorylated forms of 2-deoxyglucose indicated that in GLUT4-expressing oocytes, under basal or insulin-stimulated conditions, most of the intracellular 2-deoxyglucose was in the phosphorylated form (near 70%) and the free/phosphorylated ratio was not modified by insulin (results not shown). These data indicate that insulin and IGF-I indeed stimulate glucose transport by GLUT4-expressing

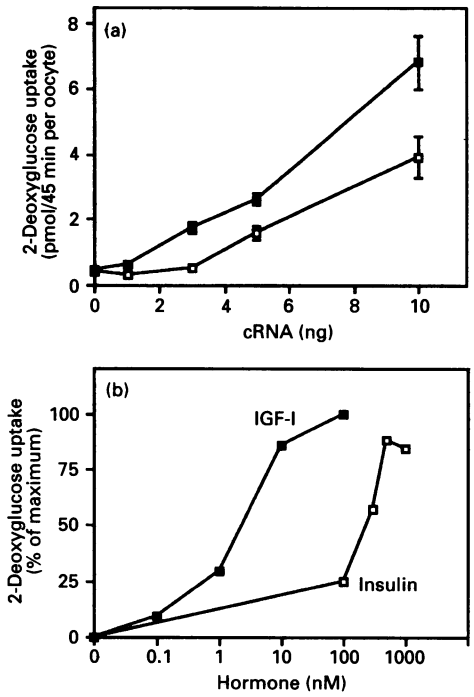

Figure 1 Characterization of the stimulatory effect of insulin and IGF-1 on 2-deoxyglucose uptake in GLUT4-expressing oocytes

(**a**) Oocytes were injected with various amounts of cRNA for GLUT4 (0–10 ng) and, 3 days after injection, 2-deoxyglucose uptake was determined. On the experimental days, oocytes were incubated for 60 min in the absence (□) or presence (■) of 1 μM insulin. (**b**) Dose-dependence of insulin and IGF-I on the stimulation of 2-deoxyglucose uptake in GLUT4-expressing oocytes. Oocytes were injected with 10 ng of cRNA for GLUT4 and, 4 days after injection, 2-deoxyglucose uptake was determined. On the experimental day, oocytes were incubated for 60 min in the absence or presence of different concentrations of insulin (□; 0–1 μM) or IGF-I (■; 0–100 nM). 2-Deoxyglucose uptake was determined for 45 min in the presence of 50 μM 2-deoxy[^{3}H]glucose at 5 μCi/ml. Results are means ± S.E.M. obtained from seven or ten oocytes per group of a representative experiment.

oocytes. To assess further whether hormones stimulate the activity of GLUT4 carriers, experiments were performed in which oocytes were injected with different amounts of GLUT4 cRNA and, therefore, oocytes expressed various amounts of GLUT4 (Figure 1). At 3 days after cRNA injection, oocytes were incubated in the absence or presence of insulin, and 2-deoxyglucose uptake was determined. Under basal, unstimulated, conditions, and in keeping with previous observations, 2-deoxyglucose uptake was dependent on the amount of GLUT4 cRNA injected (Figure 1a). Futhermore, the absolute increase in 2-deoxyglucose uptake due to insulin stimulation was dependent on the amount of GLUT4 cRNA injected, so the maximal stimulation was observed after the injection of 10 ng of GLUT4 cRNA (Figure 1a). Furthermore, the effect of insulin and IGF-I on 2-deoxyglucose uptake occurred in a concentration-dependent manner in the oocytes that were injected with 10 ng of GLUT4 cRNA (Figure 1b). The ED_{50} of IGF-I for this effect was nearly 100-fold more potent than that of insulin (Figure 1b). The dose–response curves of IGF-I and insulin effects on 2-deoxyglucose uptake are similar to the dose–response curves previously reported for IGF-I- and insulin-induced oocyte maturation [21,37].

In all, these results indicate that insulin and IGF-I specifically stimulate the activity of glucose transport in GLUT4-expressing oocytes by activation of the IGF-I-receptor, and in a manner

Table 2 Synergistic effects of insulin and 1F8 on the 2-deoxyglucose uptake in GLUT4-expressing oocytes

Oocytes were injected with water or with 10 ng of cRNA for GLUT4. At 5 days after the injection, water-injected oocytes were injected with water or with monoclonal antibody 1F8. GLUT4-expressing oocytes were injected with water (GLUT4), with monoclonal antibody 1F8 (GLUT4 + 1F8) or with monoclonal antibody anti-VAMP (GLUT4 + Cl 10.1). Then 60 min later, oocytes were incubated in the absence or presence of 1 μM insulin for an additional 60 min. 2-Deoxyglucose uptake was determined for 45 min in the presence of 50 μM 2-deoxy[^{3}H]glucose at 5 μCi/ml. Results are means ± S.E.M. obtained from seven or ten oocytes per group of a representative experiment; * indicates a significant difference due to insulin treatment, at $P < 0.05$; † indicates a significant difference from the GLUT4 group, at $P < 0.05$.

	2-Deoxyglucose uptake (pmol/45 min per oocyte)	
	Basal	Insulin
Water-injected	0.68 ± 0.06†	0.83 ± 0.04†
1F8	0.79 ± 0.11†	0.83 ± 0.05†
GLUT4	1.1 ± 0.1	3.4 ± 0.3*
GLUT4 + 1F8	5.4 ± 0.6†	18.0 ± 1.5†
GLUT4 + Cl 10.1	1.6 ± 0.3	3.2 ± 0.4*

dependent on the level of expression of GLUT4 protein, which indicates that the target of insulin action was GLUT4 itself. There is some controversy about the sensitivity of the oocyte glucose transport machinery to insulin. Thus, some authors have reported an insulin-induced stimulation of glucose transport in intact oocytes [21,23] or in oocytes expressing different mammalian glucose-carrier isoforms [22,38]. In contrast, there are other reports in which no effects of insulin have been found after heterologous expression of glucose carriers [33,39]. Under our experimental conditions, we found an insulin-induced glucose transport activation only in GLUT4-expressing oocytes. In this regard, it should be pointed out that we have obtained stimulatory effects of insulin on 2-deoxyglucose uptake in more than 20 independent experiments performed over 2 years, suggesting that the effects of insulin and IGF-I are independent of annual rhythms. Whether the variable response to insulin found in the literature depends on the nutritional or environmental conditions of the *Xenopus* frogs, the endogenous status of the oocytes subjected to analysis, or the level of glucose transporters when heterologously expressed in oocytes, remains unknown.

To obtain further insight into the nature of the stimulatory effect of insulin on glucose transport in GLUT4-expressing oocytes, we took advantage of the recent observation that injection of antibodies directed against the C-terminus of GLUT4 into *Xenopus* oocytes stimulates GLUT4 activity by changing the K_m value for glucose [40]. In keeping with these observations, we found that monoclonal antibody 1F8 [5], which recognizes the C-terminus of GLUT4, upon injection in GLUT4-expressing oocytes (21 ng/oocyte) caused a 5-fold stimulation of 2-deoxyglucose uptake. In contrast, 1F8 had no effect in water-injected oocytes (Table 2). In addition, monoclonal antibody 1F8 and insulin activated glucose transport in GLUT4-expressing oocytes in a synergistic manner (1F8 injection, insulin or the combination of 1F8 and insulin caused a 5-, 3- or 17-fold increase respectively in 2-deoxyglucose uptake) (Table 2). However, injection of monoclonal antibody Cl 10.1 anti-VAMP did not mimic the effect of antibody 1F8 in either the absence or the presence of insulin (Table 2). These observations strongly suggest that 1F8 stimulates more plasma-membrane GLUT4 carriers in insulin-treated oocytes than in oocytes not treated with insulin.

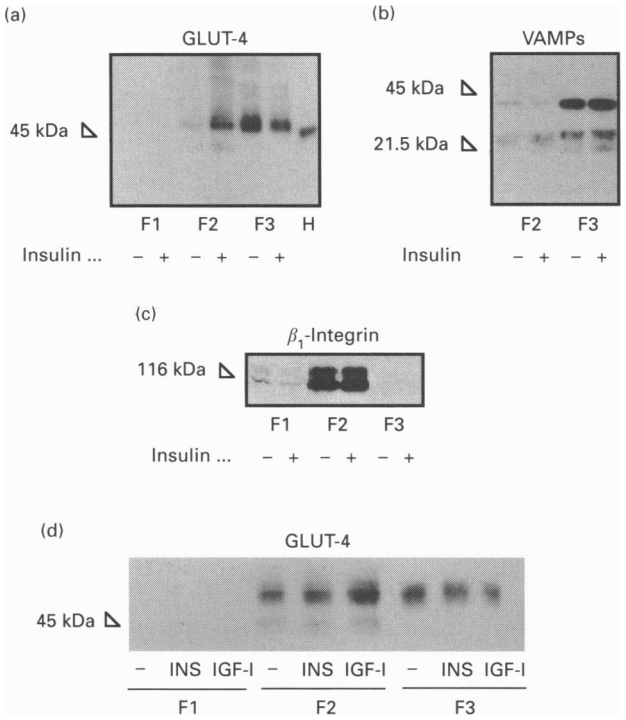

Figure 2 Insulin and IGF-I recruit GLUT4 to the plasma membrane of the *Xenopus* oocyte

Oocytes were injected with 10 ng of cRNA for GLUT4. At 5 days after the injection, oocytes were incubated for 60 min in the absence (−) or in the presence (+) of 1 μM insulin (**a**, **b**, **c**). In some other experiments, oocytes were incubated for 60 min in the absence (−) or presence (+) of 1 μM insulin (INS) or 100 nM IGF-I (**d**). Oocytes were then homogenized, and three different membrane fractions were obtained: F1, F2 and F3. A 10 μg sample of each was subjected to Western-blot analysis using specific antibodies directed against the C-terminus of GLUT4 (**a** and **d**), against *Xenopus* β_1-integrin (**c**) and against rat VAMP (**b**). Autoradiograms were subjected to scanning densitometry. Representative autoradiograms from five to seven separate experiments are shown. The positions of 21.5 kDa, 45 kDa and 116 kDa markers are shown to the left of the panels.

Insulin and IGF-I translocate GLUT4 to the cell surface of oocytes

To obtain direct evidence for an insulin-induced translocation of GLUT4 in GLUT4-expressing oocytes, we performed subcellular fractionation of the oocyte in order to separate plasma membrane from intracellular membranes. To this end, we followed the method reported by Thomas et al. [34], which leads to the isolation of three distinct membrane fractions, i.e. F1 mainly enriched in melanosomes, F2 enriched in plasma membranes and F3 enriched in intracellular membrane components. These membrane fractions were characterized on the basis of the abundance of β1-integrin, a plasma-membrane marker, and VAMP, a protein involved in vesicle recycling, as a marker of intracellular membranes. In keeping with previous data, fraction F2 was highly enriched in plasma-membrane components, as judged by the abundance of β_1-integrin (Figure 2c). [Some β_1-integrin was also detected in fraction F1, suggesting that this fraction also contained plasma membranes (Figure 2c).] The presence of VAMP-like proteins was determined by using a monoclonal antibody against rat VAMP [26]. Three distinct bands were immunodetected in oocyte extracts with anti-(rat synaptobrevin) serum, with apparent molecular masses of 36, 23 and 19 kDa (Figure 2b). Furthermore, VAMP-like proteins were essentially restricted to fraction F3, indicating that F3 had an intracellular origin. Under non-stimulated conditions, GLUT4 was most abundant in the intracellular fraction F3 (Figure 2a), and calculation of the amount of GLUT4 per total fraction indicated that 12% of cellular GLUT4 was in the plasma membrane. In contrast, GLUT1 was most abundant in the plasma-membrane fraction F2, compared with its presence in F1 or F3 in GLUT1-expressing oocytes (results not shown).

Incubation of GLUT4-expressing oocytes ($n = 20$) in the presence of insulin did not modify the yield of membrane proteins obtained from plasma membranes (F2 fraction, 96 ± 7 μg and 79 ± 15 μg) and intracellular membranes (F3 fraction, 590 ± 102 μg and 512 ± 130 μg). The abundance of β_1-integrin in the plasma-membrane fraction was indistinguishable when control and insulin-treated groups were compared (Figure 2c). Under these conditions, a 2.6-fold increase in the abundance of GLUT4 was detected in plasma membrane from insulin-treated oocytes (Figure 2a). This was concomitant to a 26% decrease in the abundance of GLUT4 in intracellular membranes due to insulin treatment (Figure 2). No change in the abundance of VAMP-like proteins was detected between control and insulin-treated groups (Figure 2b). A similar effect of IGF-I causing the translocation of GLUT4 from an intracellular membrane fraction to the plasma membrane was also detected (Figure 2d).

These studies reveal an insulin- and IGF-I-induced recruitment of GLUT4 carriers from a membrane fraction enriched in intracellular membranes to a membrane fraction highly enriched in plasma-membrane components. Furthermore, these results indicate that GLUT4 carriers, when expressed in *Xenopus* oocytes, maintain the capacity to be recruited to the cell surface in response to insulin or IGF-I. The mechanism by which GLUT4 carriers expressed in oocytes can be recruited by insulin, and why this effect cannot be detected in mammalian insulin-insensitive cells such as fibroblasts [13,14], Chinese hamster ovary cells [17] or C2C12 muscle cells [16] after transfection with GLUT4, remain to be determined.

Wortmannin blocks the effect of insulin on PI 3-kinase activity and on glucose transport in GLUT4-expressing oocytes

Recent data support the view that PI 3-kinase activity, which is stimulated by insulin [29,41] during association with tyrosine-phosphorylated IRS-1 [42], might have a role in the stimulatory effect of insulin on glucose transport. Available information indicates that wortmannin, a potent inhibitor of PI 3-kinase [43,44], inhibits the effect of insulin on glucose transport activity and on GLUT4 glucose-carrier translocation in adipocytes and CHO cells [45–47]. The hypothesis that PI 3-kinase activity might regulate vesicle trafficking is further supported by the fact that the PI 3-kinase encoded by yeast VPS34 gene is essential for protein sorting [48] and that wortmannin also inhibits histamine release from basophilic leukaemia cells [44]. To obtain some insight into the insulin-signalling pathways involved in the stimulatory effect on GLUT4 in oocytes, we studied the effects of wortmannin on PI 3-kinase activity and insulin-stimulated glucose transport. Oocytes were incubated with insulin, and anti-phosphotyrosine immunoprecipitates were prepared from a solubilized fraction of the whole cell lysate and assayed for PI 3-kinase activity (Figure 3). Insulin at a concentration of 1 μM caused an increase in anti-phosphotyrosine-immunoprecipitable PI 3-kinase activity (Figure 3). When intact oocytes were treated with insulin and 1 μM wortmannin, a marked decrease in PI 3-kinase activity was detected (Figure 3). The product of PI 3-kinase reaction catalysed on the anti-phosphotyrosine immunoprecipitates was only PI-3-phosphate; no PI-4-P was generated during the incubation, indicating that the immunoprecipitates were not contaminated with PI 4-kinase (results not shown).

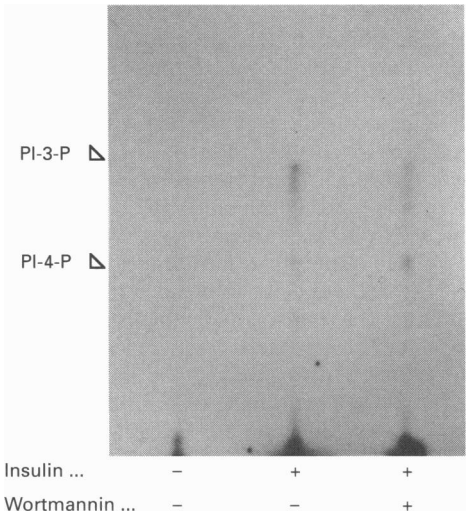

Figure 3 Wortmannin inhibits the effect of insulin on PI 3-kinase in *Xenopus* ooxytes

Oocytes were incubated in the absence or in the presence of 1 μM insulin or 1 μM wortmannin. Thereafter, PI 3-kinase activity was measured in anti-(phosphotyrosine kinase) immunoprecipitates incubated with [γ-^{32}P]ATP and PI. Autoradiograms were subjected to scanning densitometry. The data shown correspond to a representative experiment.

Table 3 Wortmannin does not alter basal 2-deoxyglucose uptake in water-injected and GLUT4- and GLUT1-expressing oocytes

Oocytes were injected with water or with 10 ng of cRNA for GLUT4 or GLUT1. At 5 days after the injection, oocytes were incubated in the presence of various concentrations of wortmannin, and 30 min later 2-deoxyglucose uptake was determined (see the legend to Figure 1). Results are means ± S.E.M. obtained from seven or ten oocytes per group of a representative experiment.

	2-Deoxyglucose uptake (pmol/45 min per oocyte)		
Wortmannin (nM)	Water-injected	GLUT4	GLUT1
0	0.82 ± 0.03	1.83 ± 0.35	67.4 ± 4.7
10	0.94 ± 0.04	1.95 ± 0.23	64.4 ± 4.8
50	0.82 ± 0.05	1.95 ± 0.25	69.7 ± 2.8
1000	0.71 ± 0.02	1.83 ± 0.51	67.5 ± 3.4

The presence of different concentrations of wortmannin did not alter the basal rate of 2-deoxyglucose uptake in water-injected, GLUT4-expressing and GLUT1-expressing oocytes (Table 3). However, wortmannin inhibited the stimulatory effect of insulin on glucose transport activity in GLUT4-expressing oocytes (Figure 4); half-maximal inhibition was observed at a concentration of approx. 10 nM.

These observations suggest a critical role of PI 3-kinase in the signalling pathway of insulin that leads to GLUT4 activation in oocytes. Our data indicate that (a) similar mechanisms might be involved in the activation of GLUT4 translocation in murine adipocytes and in *Xenopus* oocytes, and (b) PI 3-kinase is also critical in the stimulatory effect on glucose transport triggered by activation of the IGF-I receptor. This latter aspect is consistent with previous observations indicating that IGF-I receptor via IRS-1 also associates with and activates PI 3-kinase [48,49]. On the basis of these findings, we conclude that the heterologous

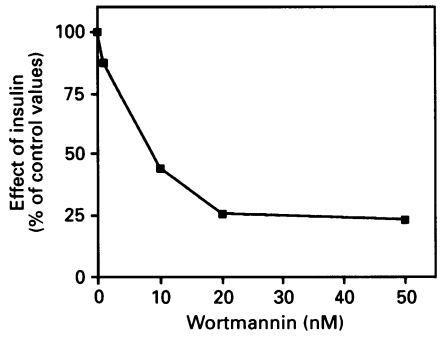

Figure 4 Wortmannin inhibits the effect of insulin on 2-deoxyglucose uptake in GLUT4-expressing oocytes

Oocytes were injected with 10 ng of cRNA for GLUT4. At 5 days after the injection, oocytes were incubated in the presence of various concentrations of wortmannin and, 30 min later, oocytes were also incubated in the absence or in the presence of 1 μM insulin for an additional 60 min (water-injected oocytes were only incubated in the absence of insulin), and then 2-deoxyglucose uptake was determined. Results of the stimulatory effect of insulin on glucose transport were expressed as a percentage of control values (no wortmannin).

expression of GLUT4 in *Xenopus* oocytes is a useful experimental approach by which to study the specific pathway/s of insulin signalling involved in the activation of GLUT4.

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